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Breast cancer is a disease where breast epithelial cells become refractory to appropriate growth and differentiation signals. It is likely that numerous genetic changes can contribute to malignant transformation, including mutations that alter the cell cycle regulatory machinery. We have therefore sought to characterize the function of both positive and negative cell cycle regulatory elements in normal and malignant breast epithelial cells. In particular, we have focused on cyclin E, a positive cell CYCLE REGULATOR ELEMENT ALREADY IMPLICATED IN SOME BREAST MALIGNANCIES and on a class of negative regulators of cyclin-dependent kinases (Cdks). Preliminary data indicate that both cylin E and Cdk inhibitors are important in breast malignancy.

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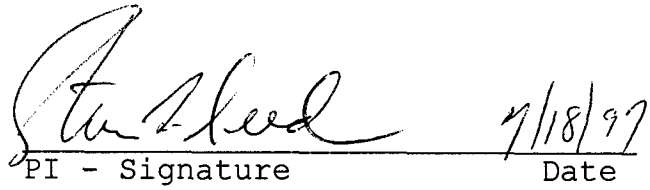

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(5) INTRODUCTION

Breast cancer is a disease where breast epithelial cells become refractory to growth and differentiation signals. It is likely that numerous genetic changes can contribute to malignant transformation, including mutations that alter the cell cycle regulatory machinery. We have therefore sought to characterize the function of both positive and negative cell cycle regulatory elements in normal and malignant breast epithelial cells. In particular, we have focused on cyclin E, a positive cell cycle regulatory element already implicated in some breast malignancies and a class of negative regulators of cyclin dependent kinases (Cdks). To study the role of cyclin E in breast malignancy, we screened cell lines derived from breast carcinomas for cyclin E mutations. We have also created cyclin E mutations and studied their effects on cultured cells. Additionally, we have targeted cyclin E using antisense strategies to examine its essentiality. To investigate the role of Cdk inhibitors in regulation of proliferation of breast epithelial cells, we have (1) identified and studied Cdk inhibitors in other cell types where analysis is more straightforward and (2) attempted to extrapolate these results to breast epithelial cells to determine the relevance for cell cycle control in this system.

(6) BODY

(a) SOW Task 1: Characterization of Cdk inhibitor production in normal and malignant breast epithelial cells (months 1-24).

This project was terminated as of the previous report.

(b) SOW Task 2: Characterization of cyclin/Cdk complexes in normal and malignant breast epithelial cells (months 1-36).

This project was completed/terminated as of the previous report.

(c) SOW Task 3: Test of essentiality of cell cycle regulatory components in breast epithelial cells by antisense (months 1-12).

We planned to use three antisense methods to test for essentiality of cyclin E and, eventually, other proteins: antisense deoxyoligonucleotides, antisense ribozymes and antisense-encoding adenoviruses. Although initial results using oligonucleotides were discouraging, we have now obtained reasonable results using C-5 propyne-modified pyrimidine substituted oligonucleotides (1). Fig. 1 shows a cyclin E western blot corresponding to non-transformed human breast epithelial cells (184A1) synchronized by withdrawal and replenishment of EGF and treated with cyclin E antisense and control oligonucleotides. As can be seen, the antisense oligonucleotide significantly reduces the level of cyclin E relative to the control oligonucleotide at all time points. However, FACS analysis of the same cells indicated that severe reduction in cyclin E levels was having little, if any, impact on rate of entry into S phase or progression through the cell cycle (Fig. 2). Therefore, it appears that cyclin E levels are not rate-limiting for entry into S phase in breast epithelial cells. However, when immunoprecipitation cyclin E/Cdk2 kinase assays were performed on the same lysates, it was clear that even though the antisense treated lysates had much less cyclin E than controls (<20%), the cyclin E-associated kinase activity was similar (Fig. 3). Thus, the specific activity of the kinase was elevated to compensate for the reduction in cyclin E levels. To understand the

mechanism for this compensatory adjustment we evaluated the presence of the inhibitor p27 and the phosphorylation status of Y15 of cyclin E-associated Cdk2. Although the levels of p27 were similar in the antisense and control samples, the phosphorylation level of Y15 of cyclin E-associated Cdk2 was greatly reduced in the antisense-treated samples. Thus it appears that the specific activity of Cdk2 in cells deprived of cyclin E is modulated at the level phopsphorylation of Y15. We are currently investigating this at the mechanistic level.

Methods: An oligonucleotide (15-mer) was synthesized corresponding to nucleotide 543 to 557 of the cyclin E cDNA was transfected into asynchronous 184A cells using cationic lipid. Cells were either harvested to prepare protein for Western blotting or for FACS analysis at 24 hours post transfection.

In the hope of obtaining a more complete elimination of cyclin E, we attempted to pursue the antisense ribozyme approach. This not only proved technically difficult but it has become clear that ribozymes do not cleave at a sufficient catalytic rate to be useful in vivo against short half-life mRNAs. Using the random antisense library approach of Lieber and Strauss (2), a strong, ribozyme-accessible cleavage site was found in cyclin E mRNA. This ribozyme could cleave cyclin E RNA in vitro but had little effect in vivo.

Methods: standard molecular biology procedures were employed.

In addition to ribozymes, we tried to utilize antisense expressing recombinant defective adenoviruses as an alternative approach. This method has the advantage of achieving regulated high uniform levels of antisense expression in total populations of cells. Recombinant adenovirus stocks expressing the complete

cyclin E cDNA in antisense orientation were prepared and transduced into 184A1 breast epithelial cells to assess the effects on cyclin E levels and to determine phenotype. However, 184A1 cells do not infect well with adenovirus and significant effects were not observed (Fig. 4).

Methods: Recombinant adenoviruses are prepared by co-transfecting a plasmid containing an expression cassette embedded in adenovirus sequences with a fragment containing most of the adenovirus genome into 293 cells, which can complement the missing viral functions. Recombinant viruses are placque purified, amplified and purified by cesium chloride banding.

Since several reports suggest that abnormal accumulation of cyclin E may have a role in the etiology of breast cancer (3,4), we have also taken the liberty of investigating the physiology of cyclin E accumulation. Even though this was not explicit in the original proposal, we feel that it is well within the scope of the project.

We identified cyclin E mutations that lead to hyperaccumulation of cyclin E, analysis of which indicated that cyclin E ubiquitination and turnover are regulated by autophosphorylation of cyclin E/Cdk2 on Thr380 of cyclin E (5). Mutating this residue leads to increased half-life and persistent accumulation of cyclin E in mammalian cells (Fig. 5). We have now shown that the consequences of this is perturbed progression through S phase (Fig. 6) and, more importantly, genetic instability. Conditional expression of stabilized mutant alleles of cyclin E in Rat-1 fibroblasts, allows enhanced chromosome loss (Fig. 7). We are now performing similar experiments in 184A human breast epithelial cells. We have also set up an SSCP analysis for analyzing the C-terminus of the cyclin E coding region in DNA from tumor biopsies to determine of stabilizing mutations in cyclin E might be

associated with breast cancer. So far analysis of 20 biopsies has yielded a polymorphism but no mutations.

Methods: Standard molecular biological approaches were employed. Mutant and wild type cyclin E alleles were expressed in Rat-1 fibroblasts and are being expressed in 184A breast epithelial cells using the tetracycline repressible system (6). Rate of progression through S phase was determined by BUdR pulse-labelling followed by a thymidine chase time course and analysis by FACS. Chromosome loss was measured by growing cells in the presence or absence of tetracycline for 4 weeks, photographing mitotic chromosome spreads, and directly counting the number of chromosomes per spread.

(d) SOW Task 4: Cloning and characterization of Cdk inhibitors from HeLa cells (months 1-36).

As reported previously, we characterized a Cdk inhibitor activity from HeLa cells and demonstrated that it corresponded to $p27^{Kip1}$. Analysis of the regulation of p27 in HeLa cells and in normal human diploid fibroblasts indicated that p27 accumulation in response to drugs was mediated by translational control (7). p27 accumulation in response to contact inhibition was shown to be mediated both at the level of translational control as well as modulation of the half-life of the protein (7). In neither instance was transcriptional control implicated.

In order to analyze a potential role of the long 5' untranslated region (UTR) of the p27 mRNA in translational control, the 5'UTR was fused to a luciferase reporter and placed under the control of the CMV promoter. As a control, a similar

reporter construct without the p27 5'UTR was assembled. Parallel transfections were performed into HeLa cells. Clones containing the p27 5' UTR produced a significantly elevated level of reporter expression relative to the controls (Fig. 8), suggesting that this RNA sequence contains a translational enhancer. Donwregulation of p27 translation most likely involves the neutralization of this enhancer activity via the binding of proteins in trans. We are currently trying to identify the cis-acting sequences and trans-acting proteins involved in this regulation.

Methods: The complete 5'UTR of human p27 was cloned and sequenced by standard molecular biological methods. Clones for expression were constructed by standard molecular biological techniques. Mammalian cell transfections and luciferase assays were performed using standard methods.

(e) SOW Task 5: Cloning and characterization of breast epithelial cell Cdk inhibitors (months 12-48).

We have not been able to identify any new Cdk inhibitors specifically associated with breast epithelial cells. However, since p27 was cloned under the auspices of this project, we have decided to further charcterize the mechanism of p27 action (8). We have found that a single molecule of p27 is sufficient to inhibit a single complex of cyclin A/Cdk2 more than 95% (Fig. 9), thus ruling out the necessity for greater than 1:1 stoichiometry, as has been proposed for the related inhibitor p21. We are currently investigating the inhibitory stoichiomtry of p27 on other Cdk complexes such as cyclin D1/Cdk4.

Methods: p27 expressed in E. coli and cyclin A/Cdk2 expressed in insect cells were purified by standard biochemical techniques. cyclin A/Cdk2 kinase activity was measured using histone H1 and labelled ATP as substrates. Kinase activity was compared when fixing cyclin A/Cdk2 to beads via p27 vs. via anti-cyclin A antibodies.

(f) SOW Task 6: Characterization of Cdk inhibitors in vivo (months 24-48). This task has been obviated since no new Cdk inhibitors were identified.

(7) CONCLUSIONS

We could provide no compelling evidence that our initial hypothesis that Cip/Kip family Cdk inhibitor proteins were important for regulation of the cell cycle in human breast epithelial cells. Although these cells express p21 and p27 to some degree, they do not seem to be implicated directly in the regulatory modes that we investigated.

Since we cloned human p27 in the context of this project, we have continued to investigate its regulation. In particular, we have determined that translational control rather than transcriptional control is the primary mode of regulation in many different cell cycle regulatory contexts. Correlative data suggest that regulation of ribosomal function by pp70 S6 kinase may be involved, although the regulation of p27 translation by this kinase would be the converse to what has been demonstrated for other translationally-regulated mRNAs so far. In addition, analysis of the 5' UTR of the p27 mRNA is consistent with possible translational regulation. In initial experiments, it appears that the 5'UTR of the p27 mRNA contains a translational enhancer, leading to the hypothesis that translational

control is mediated via inactivation of this enhancer, most likely by the binding of transactivating factors.

A major effort is now being focused on understanding the function and regulation of cyclin E. Antisense work has suggested that accumulation of cyclin E protein is not normally rate-limiting for the G1 to S phase transition in breast epithelial cell lines, which runs counter to accepted dogma for mammalian cells, in general. However, we have found that cells compensate for low cyclin E levels by increasing the specific activity of cyclin E/Cdk kinase. This is achieved by modulating the level of Y15 inhibitory phosphorylation on Cdk2. Therefore, the lack of a cell cycle effect observed results from the cells' ability to maintain Cdk kinase levels even under conditions of limiting cyclin E. Our attempts to use antisense ribozymes and antisense adenovirus approaches for targeting cyclin E have proven unworkable due to inherent limitations of these technologies. However, we are experimenting with improved antisense oligonucleotide technologies in the hope of more completely limiting cyclin E in vivo in breast epithelial cells.

A complementary issue that we are focusing on is whether abnormal accumulation of cyclin E might be important in the context of malignant transformation and breast cancer, in particular. We have found that point mutations in cyclin E can stabilize the protein dramatically in vivo and, more interestingly, lead to genetic instability in the form of dramatically increased rates of chromosome loss. Proper regulation of cyclin E levels is of particular relevance to breast cancer since transgenic mice that overexpress cyclin E in the mammary epithelium during pregnancy and lactation develop a high incidence of mammary carcinoma (4). Therefore, we are investing a signicant effort in understanding how

deregulation of cyclin E accumulation leads to loss of cell cycle control and genetic instability. We are also analyzing the cyclin E gene from tumor derived material to determine if stabilizing mutations are present, explaining, in part, the transformed phenotype.

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Figure Legends:

Figure 1. Cyclin E antisense oligonucleotides inhibit the accumulation of cyclin E in synchronized 184A1 human mammary epithelial cells. 184A1 cells were rendered quiescent by withdrawal of serum and EGF for 48 hours, transfected with C-5 propyne substituted antisense or control oligonucleotides and then stimulated to reenter the cell cycle. Cyclin E levels were monitored by Western blot as a function of time after addition of serum and EGF. Cyclin E accumulation was strongly inhibited by the antisense but not the control mismatch (Mm) oligonucleotide.

Figure 2. Reduction of cyclin E levels does not signifcantly affect entry into S phase. Flow cytometric analysis was performed on the experiment described in Fig. 1 to determine the rate of entry of stimulated cells into S phase after transfection with cyclin E antisense or control oligonucleotides. Although cyclin E accumulation was strongly inhibited by transfection with the antisense oligonucleotide, there was not signicant impact on the rate of entry into S phase.

Figure 3. Reduction of cyclin E levels does not significantly affect the associated kinase activity. Immunoprecipitation histone H1 kinase assays were performed on cyclin E/Cdk2 complexes obtained from the time course described in Fig. 1. An autoradiograph of phosphorylated histone H1 separated by SDS-PAGE indicates that although cyclin E accumulation is strongly inhibited by antisense treatment, the associated kinase activity is comparable to the control, except in quiescent cells, where cyclin E mRNA levels are much lower.

Figure 4. Cyclin E antisense adenovirus transduction has no effect on cyclin E levels in 184A1 human mammary epithelial cells. 184A1 cells were transduced with cyclin E antisense and conrol (GS-1) adenovirus at the indicated multiplicities. A cyclin E Western blot shows that no reduction in the protein was observed after 48 hours.

Figure 5. Mutation of Thr380 of human cyclin E increases its half-life in Rat-1 fibroblasts. Rat-1 clones expressing wild-type human cyclin E or a T380A mutant allele were pulse labelled with ^{35}S methionine, followed by a cold chase. Lysates were prepared at the indicated times and cyclin E was immunoprecipitated and analyzed for loss of label using a phosphorImager after SDS-PAGE. , wild-type cyclin E; , T380A.

Figure 6. Expression of a stabilized allele of cyclin E impairs progression through S phase but accelerates the G1/S phase transition. Rat-1 cells expressing cyclin E T380A under control of the tetracycline repressible promoter were pulse-labelled with BUdR in the presence or absence of tetracycline. Labelled cells were then followed as a function of time for exit from S phase and entry into the subsequent S phase. Cells expressing cyclin E T380A exited from S phase more slowly but entered the next S phase more rapidly the cells where expression was repressed by tetracycline. -, no induction; +, induction; n-, no induction, next S phase; n+, induction, next S phase.

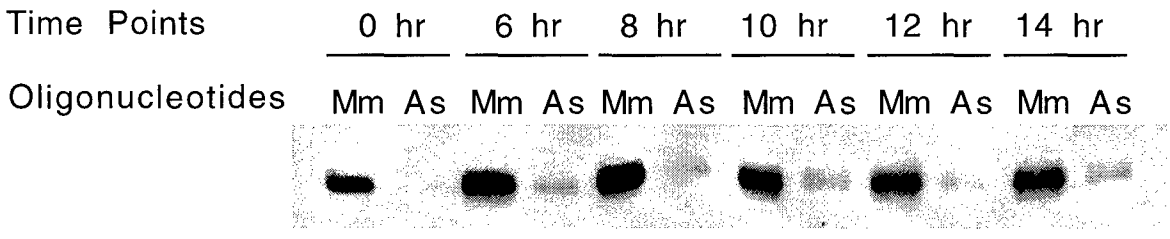
Figure 7. Expression of a stabilized allele of cyclin E in Rat-1 cells leads to elevated levels of chromosome loss. Cells expressing cyclin E T380A under control of the tetracycline repressible promoter were cultured for four weeks either in the presence or absence of tetracycline. Mitotic chromosome spreads were then prepared. The histogram represents the data from total chromosome counts for

fifty mitotic spreads +/- tetracycline. Based on these data, approximately one out of three cells expressing the mutant cyclin E allele lost a chromosome.

Figure 8. A comparison of expression of luciferase from constructs with (p27) and without (pGL2) the p27 5'UTR. Transient transfections were performed using HeLa cells. After transfection, cells were left growing asynchronously (Asyn.), arrested in S phase using a single thymidine block or arrested in G1 using the drug lovastatin. Luciferase expression was measured using a luminometer. Clearly, the 5'UTR of p27 leads to elevated expression of luciferase under all conditions. However, this sytem doesn't recapitulate translational regulation, i.e. higher expression in lovastatin and lower expression in an S phase block, presumably because the mRNA is overexpressed saturating the regulatory capacity of the system.

Figure 9. A single molecule of p27 inhibits a single cyclin A/Cdk2 complex. (A) p27 was fixed to anti-p27 beads in two amounts (1x, lane 1 or 1/4x, lane 3) and used to adsorb a fixed amount of purified recombinant cyclin A/Cdk2. 1x bead/cyclin A/Cdk2 complexes, in addition, were incubated with a 2x amount of p27, prior to assay (lane 2). cyclin A/Cdk2 was also adsorbed to anti-cyclin A beads. Histone kinase assays were performed (top panel). (B) Counts determined using a PhosphorImager were normalized to precipitated Cdk2 (middle panel of A). It is clear that a single molecule of p27 inhibits a cyclin A/Cdk2 complex more than 95%.

Figure 1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6 hr
8 hr
10 hr
12 hr
14 hr
Oligonucleotides
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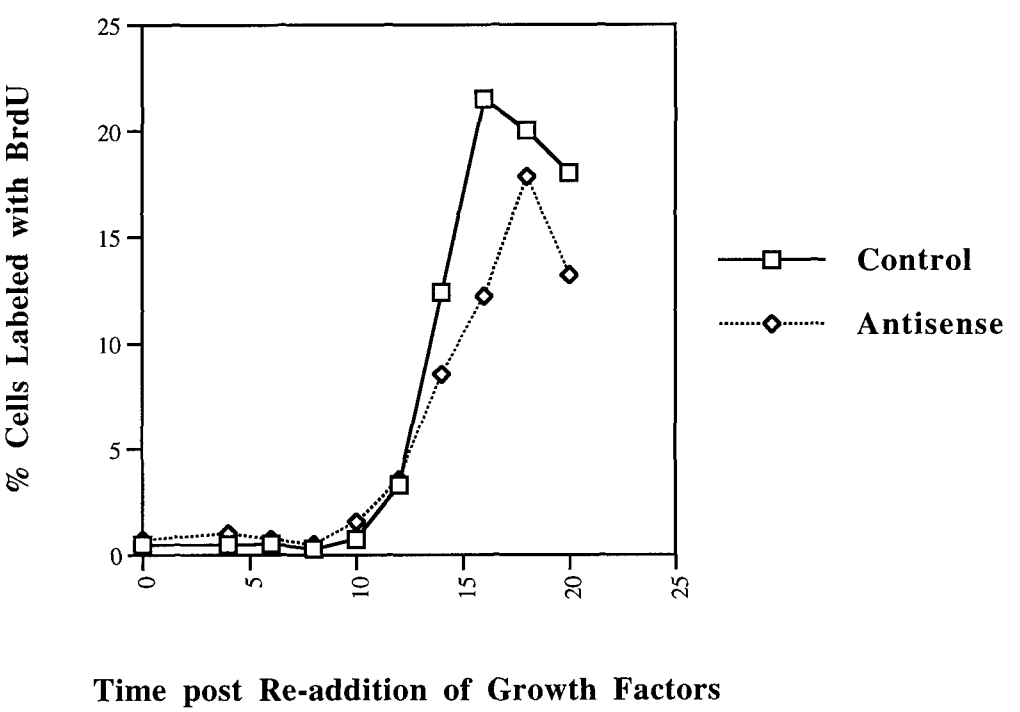
Figure 2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10
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5
10
15
20
25
Control
Antisense
Time post Re-addition of Growth Factors

Figure 3

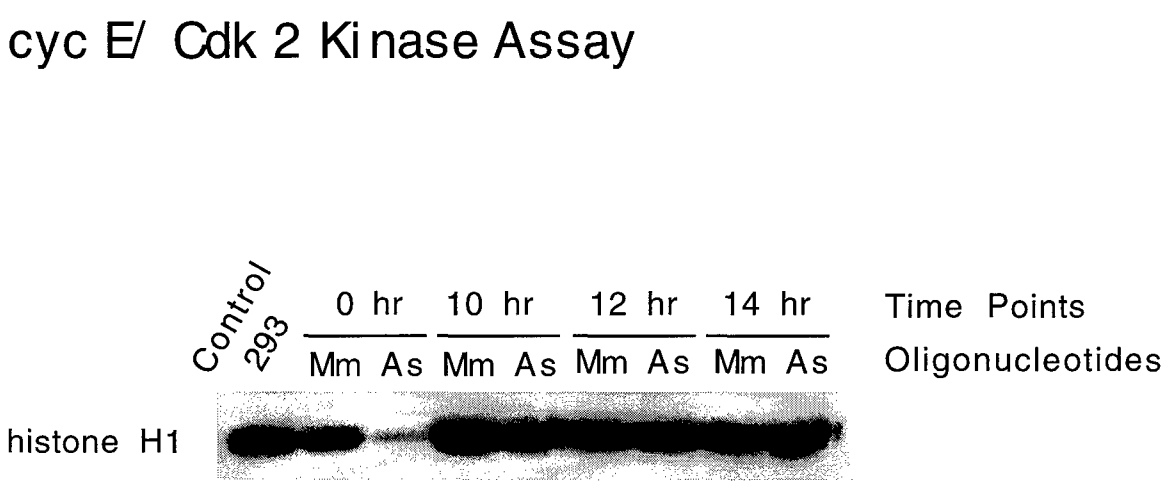
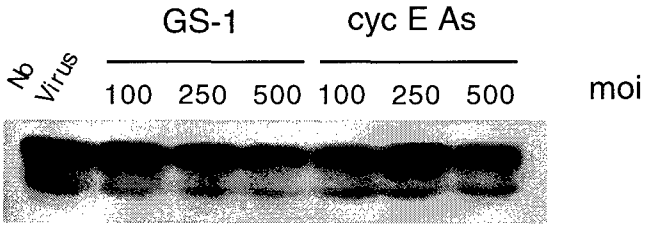

cyc E/ Cdk 2 Kinase Assay
Control 293
0 hr
10 hr
12 hr
14 hr
Time Points
Mm As Mm As Mm As Mm As
Oligonucleotides
histone H1

Figure 4

Western Blot Analysis of cyc E Expression in AdCMVcycE(AS) Infected 184A1-1 Cells



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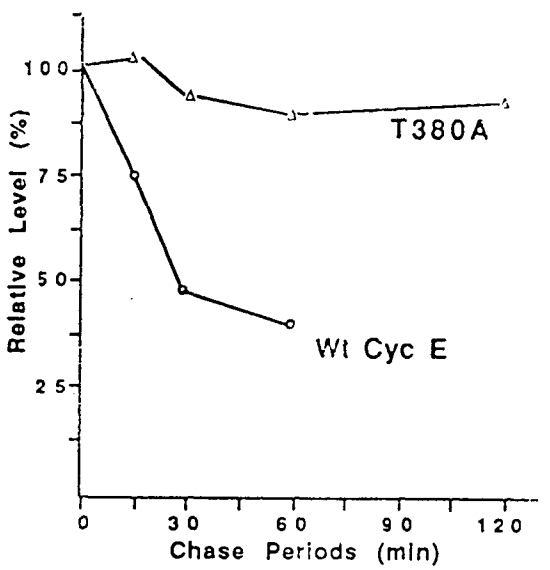
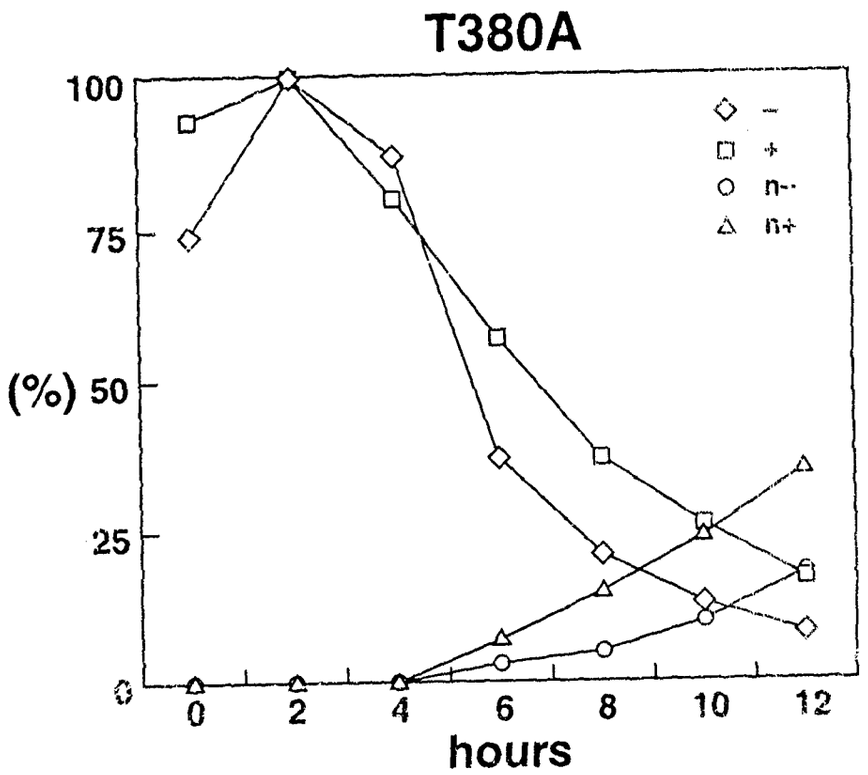

100
75
50
25
Relative Level (%)
T380A
Wt Cyc E
0
30
60
90
120
Chase Periods (min)

Figure 6



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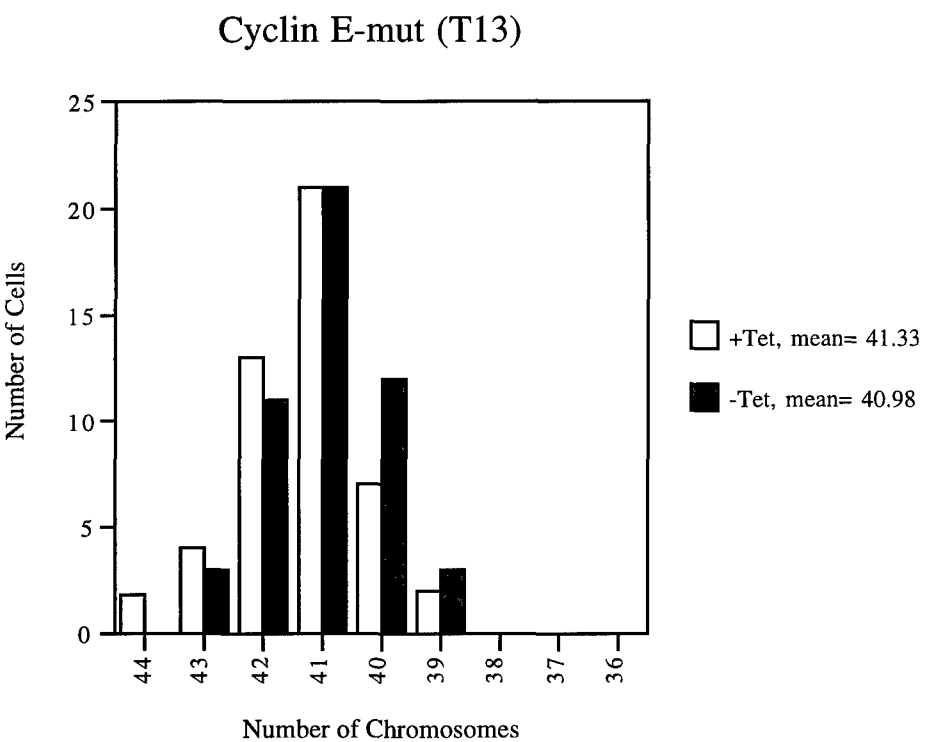
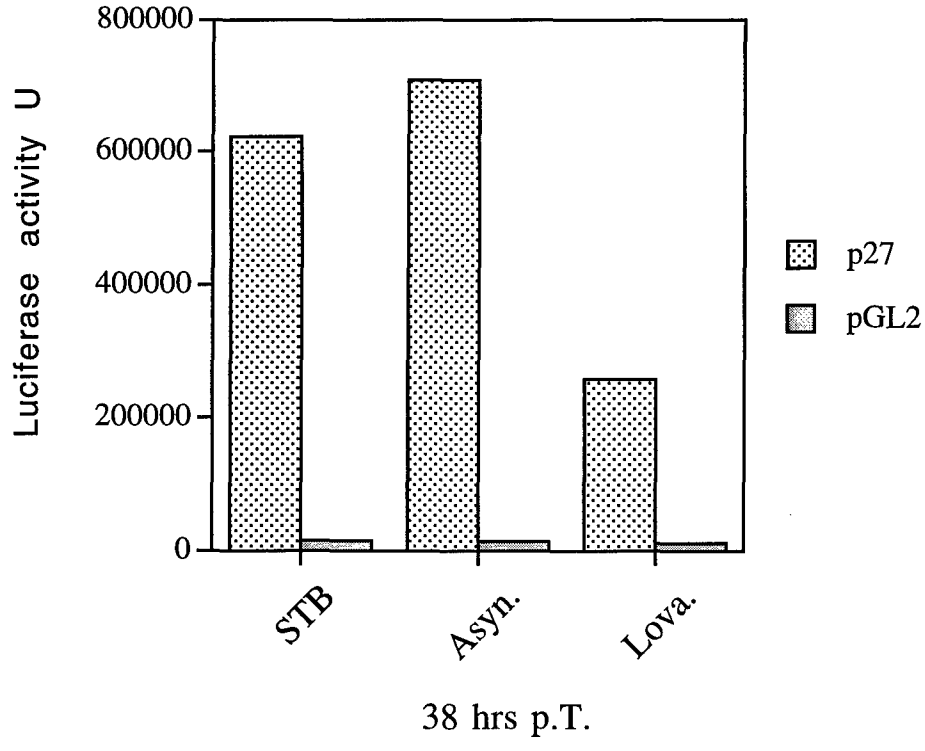

Cyclin E-mut (T13)
Number of Cells
25
20
15
10
5
0
44
43
42
41
40
39
38
37
36
Number of Chromosomes
+Tet, mean= 41.33
-Tet, mean= 40.98

Figure 8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Lova.
p27
pGL2
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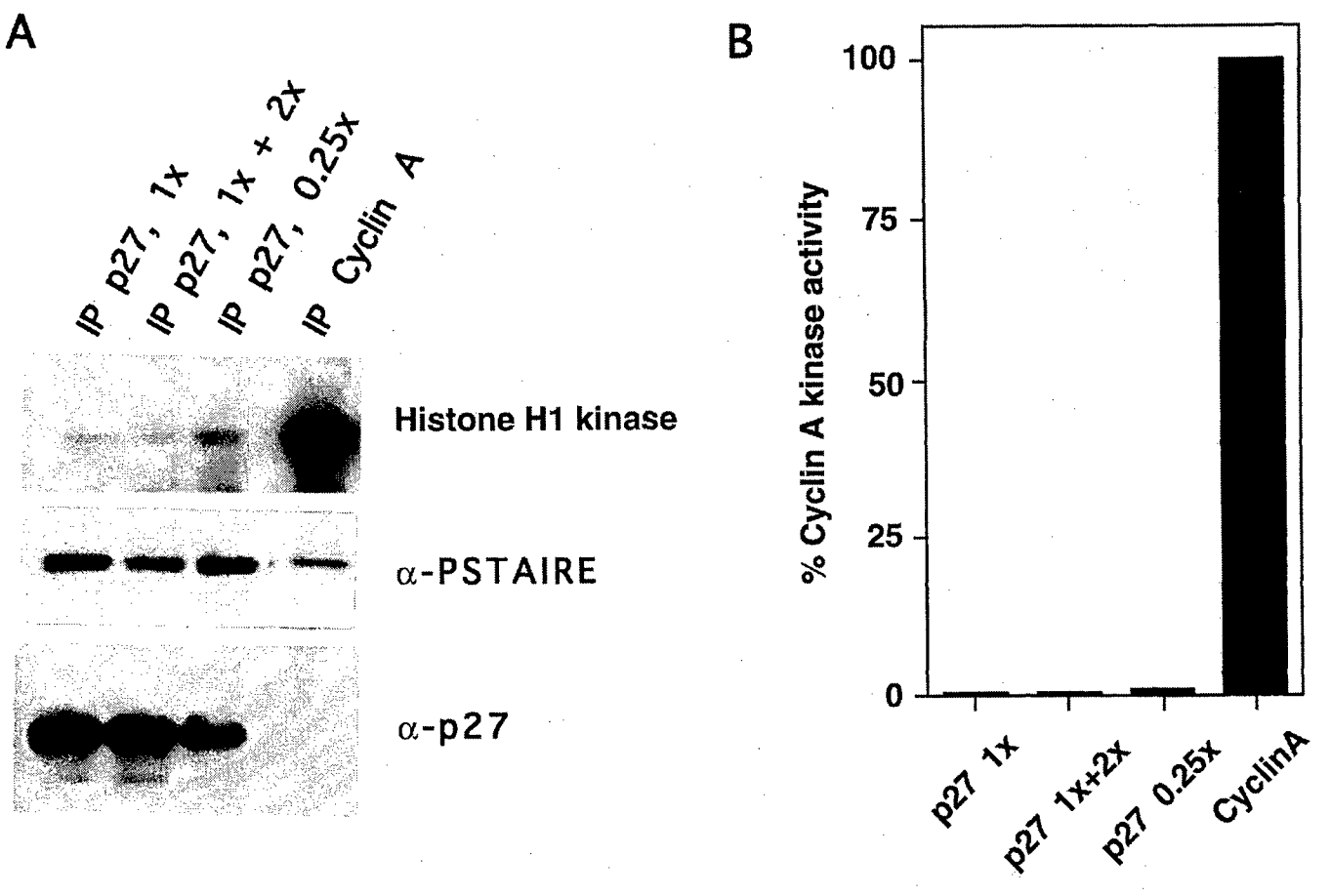
Figure 9


A
IP p27, 1x
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α-p27
B
% Cyclin A kinase activity
100
75
50
25
0
p27 1x
p27 1x+2x
p27 0.25x
CyclinA

PERSONNEL

Steven I. Reed	P.I.	19%
Meira Wolff	Technican II	100%
Kwang-Ai Won	Research Associate	100%